

# Determination of the Copy Number of Porcine Endogenous Retroviruses (PERV) in Auckland Island Pigs Repeatedly Used for Clinical Xenotransplantation and Elimination of PERV-C

[Uwe Fiebig](#) , Luise Krüger , [Joachim Denner](#) \*

Posted Date: 6 November 2023

doi: 10.20944/preprints202311.0327.v1

Keywords: porcine endogenous retroviruses; Auckland Island pigs; islet cell xenotransplantation



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Article*

# Determination of the Copy Number of Porcine Endogenous Retroviruses (PERV) in Auckland Island Pigs Repeatedly Used for Clinical Xenotransplantation and Elimination of PERV-C

Uwe Fiebig<sup>1</sup>, Luise Krüger<sup>1</sup>, and Joachim Denner<sup>1,2,\*</sup>

<sup>1</sup> Robert Koch Institute, 13353 Berlin, Germany; FiebigU@rki.de; luise.krueger@web.de

<sup>2</sup> Institute of Virology; Free University, Berlin Germany; Joachim.Denner@fu-berlin.de

\* Correspondence: Joachim.Denner@fu-berlin.de; Tel.: +491755917006, (J.D.)

**Abstract:** Auckland Island pigs represent an inbred population of feral pigs isolated on the subantarctic island for over 100 years. The animals have been maintained under pathogen-free conditions in New Zealand, they are well characterized virologically and have been used as donor sources in first clinical trials of porcine neonatal islet cell transplantation for the treatment of human diabetes patients. The animals do not carry any of the xenotransplantation-relevant viruses and in the first clinical trials no porcine viruses including porcine endogenous retroviruses (PERVs) have been transmitted to the human recipients. PERVs pose a special risk in xenotransplantation since they are part of the pig genome. When the copy number of PERVs in these animals was analyzed using droplet digital PCR and primers binding to a conserved region of the polymerase gene (PERVpol), a copy number typical for Western pigs was found, confirming previous phylogenetic analyses of microsatellites and mitochondrial analyses showing a closer relationship to European pigs than to Chinese pigs. When kidney cells from very young piglets were analyzed, only around 20 PERVpol copies were detected. Using these cells as donors in somatic cell nuclear transfer (SCNT), animals were born showing a PERVpol copy numbers between 35 and 56. This data indicates that Auckland Island pigs have a similar copy number in comparison with other Western pig breeds and that the copy number is higher in adult animals compared with cells from young piglets. Most importantly, PERV-C free animals were selected and the absence of additional eight porcine viruses was demonstrated.

**Keywords:** porcine endogenous retroviruses; Auckland Island pigs; islet cell xenotransplantation

## 1. Introduction

The Auckland Islands are a group of subantarctic islands which lie some 560 kilometers south of New Zealand. Pigs were originally introduced onto these islands in 1807 by Captain Abraham Bristow and in 1840 James Ross brought more pigs onto the island [1,2]. From the time of the last release the pig population on Auckland Island remained isolated for the next hundred years. Pigs were removed from Auckland Island in 1999 to help restore the environment to its former state and were brought to the mainland of New Zealand for conservation. 17 animals were housed, initially in quarantine, in a special, purpose-built facility in Invercargill, where they have been successfully bred [3]. The pigs are black or white to brown with black markings, have a long snout, high hair and bristle coverage for the cold conditions [1]. When the mitochondrial D-loop DNA sequences of the Auckland Island pigs were compared with sequences from domestic and wild boar, the sequence from the Auckland Island pigs clustered with domestic European breeds and they represent a single breeding population [1,2]. Phylogenetic analyses of microsatellites also showed that they are more closely related to European pigs than Chinese pigs, which is consistent with the mitochondrial analyses [3].

These pigs have been monitored for 8 years and were found negative for all tested porcine infectious agents [4–7]. Among the tested viruses potentially posing a risk for xenotransplantation were the porcine endogenous retrovirus (PERV), porcine cytomegalovirus (PCMV), a porcine roseolovirus (PCMV/PRV), porcine lymphotropic herpesvirus (PLHV), hepatitis E virus (HEV), porcine teschovirus (PTV), porcine rotaviruses A, B, C (PRVA, B, C), porcine hemagglutinating encephalomyelitis virus (PHEV), porcine parvovirus (PPV), encephalomyocarditis virus (EMCV), porcine enterovirus B (PEVB), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus (PCV). For testing diagnostic PCR and serology methods were applied. In comparison with other pig breeds, the Auckland Island pigs are the “cleanest” pigs concerning virological safety. This was the reason to use pig islets from Auckland Island pigs for the treatment of humans with diabetes. In a prospective preclinical trial, transplanting islet cells from Auckland Island pigs into non-human primates, no transmission of pig viruses was observed [6]. Most importantly, no transmission of pig viruses including PERVs was observed in two clinical trials performed in New Zealand [7] and Argentina [8].

Here we analyze the number of PERV proviruses in the genome of adult Auckland Island pigs, in cells from young piglets as well as in animals obtained by somatic cell nuclear transfer (SCNT) using these piglet cells. Furthermore, we screened these animals for an additional 8 xenotransplantation-relevant viruses, among them PCMV/PRV. PCMV/PRV is of special interest since it is known to significantly reduce the survival time of non-human primates after transplantation of PCMV/PRV-positive kidneys or hearts [9–12]. PCMV/PRV was also transmitted in the first clinical trial transplanting a pig heart into a patient in Baltimore and contributed to the death of the patient [13,14]. We confirmed the absence of PCMV/PRV and other potentially zoonotic viruses in the Auckland Island pigs.

It is known that PERV-C can recombine with PERV-A in living pigs and the resulting PERV-A/C recombinant viruses are characterized by higher virus titers compared with the paternal PERV-A [15–17]. Release of human tropic PERV-A/C from mitogen-triggered peripheral blood mononuclear cells (PBMCs) was observed in the case of different minipigs as well as ill pigs [18–22]. Since PERV-C is in contrast to PERV-A and PERV-B not present in all pigs, we screened the Auckland Island pigs for PERV-C and selected PERV-C animals for further breeding. The absence of PERV-C will prevent a recombination with PERV-A.

## 2. Materials and Methods

### 2.1. Animals and cells

Auckland Island pigs were bred by the NZ Xeno Ltd, Auckland, New Zealand as described [4,5]. DNA and blood from two groups of animals were sent to Berlin and the PERV copy numbers were analyzed. Later, primary kidney cells from four animals (two male, two female) at an age of about 4 weeks were sent to Germany and the PERV copy numbers were analyzed. Somatic cell nuclear transfer (SCNT) was performed using *in vitro* matured oocytes which were collected from abattoir derived ovaries from German Landrace/Pietrain hybrid gilts. The SCNT was performed at the Chair for Molecular Animal Breeding and Biotechnology, and Center for Innovative Medical Models (CiMM) of the Ludwig Maximilian University Munich. Prior to SCNT, matured oocytes were washed in 0.25% trypsin medium to avoid transmission of pathogens from abattoir. The embryos were transferred laparoscopically to estrus-synchronized recipient sows. 7 piglets were obtained from 373 reconstructed embryos. All piglets were clinically healthy and developed normally. Blood samples taken from 2 weeks old piglets and the foster mothers were examined for the presence of PERV-C. In addition, DNA was obtained from ear punches of the animals of the F0 generation at age 416 days and from kidney cells of the F1 generation at age of 70 days and analyzed for the PERV copy number. As an internal standard the porcine embryonic kidney cell line PK15 (ACC 640, from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell lines, Braunschweig, Germany) was used.

## 2.2. DNA isolation

DNA was extracted at XenoNZ and CiMM, DNA was isolated from blood using two DNA extraction methods: DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) or phenol chloroform extraction using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quantified using a Qubit 3.0 Fluorometer (Thermo Fischer), and the 260 nm/280 nm ratio was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Worcester, MA, USA).

## 2.3. Droplet digital PCR

Droplet digital PCR (ddPCR) was performed according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA, [<http://www.bio-rad.com/de-de/applications-technologies/droplet-digital-pcr-ddpcr-technology?ID=MDV31M4VY>]) using a QX200 droplet generator and a QX100 droplet reader (Bio-Rad) [23,24]. Purified genomic DNA (100 ng genomic DNA) was digested with MseI (New England Biolabs, Ipswich, MA, USA) (20U) at 37°C for 1 hour, and the restriction enzyme was heat-inactivated. The DNA digest was diluted to 5-10 ng/μL for the ddPCR. The ddPCR mix consisted of 10 μL 2X ddPCR Master mix, 1.8 μL of each 10 μmol/L target primers (Table 1), 0.5 μL of each 10 μmol/L fluorescent probes (FAM/HEX) (Table 1), 2.5-10 ng digested DNA, and water to a total volume of 20 μL. Samples were placed into the QX200 Droplet Generator, which utilizes proprietary reagents and microfluidics to partition the samples into 10,000-20,000 nanoliter-sized droplets. Droplets were transferred to a 96-well plate for PCR amplification in an Eppendorf Mastercycler X50 (Eppendorf, Germany). The following cycling conditions were used: 10-minute initial enzyme activation at 95°C, 40x [30 second denaturation at 94°C, 30 seconds annealing] followed by an 10 minute final elongation step at 98°C. Following PCR amplification, the samples were placed in the QX200 Droplet Reader, which analyzes each droplet individually using a two-color detection system (set to detect FAM and either HEX), enabling multiplexed analysis. The droplet reader and the QuantaSoft software count the PCR-positive and PCR-negative droplets. The fraction of positive droplets is then fitted to a Poisson distribution to determine the absolute initial copy number of the target DNA molecule in the input reaction mixture in units of copies/μL.

## 2.3. Spleen and liver tissues from non-Auckland Island pigs

Frozen samples of spleen and liver tissues from dead born (day 0), 9-30 days old and older than 12 weeks piglets from the CiMM, Munich, were used. DNA was isolated as described above and the PERV copy number was measured in both organs.

## 2.4. Tests for PERV-C

DNA from the Auckland Island pigs were used to analyze the presence of PERV-C using a conventional PCR. Primers and conditions used for were described (PCR1) [25,26] (Table 1). In addition, a real-time PCR was established using specific primers and probe (Table 1) [26]. 100 ng DNA and the SensiFAST Probe No-ROX kit (Meridian Bioscience Cincinnati, OH, USA) in a 20 μL reaction volume. The cycling conditions used were initial denaturation 5 minutes at 95°C, followed by 45 amplification cycles of 95°C for 15 seconds, and annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds in a qTOWER3 G qPCR cycler (Analytik Jena, Jena, Germany). A standard curve was produced using the 510 bp amplicon of PCR6 [26] (Table 1) as template. This PCR was performed as follows: 100 ng of DNA template (PERV-C positive pig #6249), PCR buffer I containing MgCl<sub>2</sub>, 0.2 mM dNTPs, and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Inc., Waltham, MA, USA). Each sample was subjected to an initial

**Table 1.** Primers and probes used for the estimation of the PERV copy number and the detection of PERV-C.

| Name              | Sequence                             | Location (nucleotide number) | Accession number     | Reference                                 |
|-------------------|--------------------------------------|------------------------------|----------------------|---|
| PERV pol1-forward | CGACTGCCCCAAGGGTTCAA                 | 3568-3587                    | HM159246             | Yang et al., 2015 [27]                    |
| PERV pol2-reverse | TCTCTCCTGCAAATCTGGGCC                | 3803-3783                    |                      |   |
| PERV pol probe    | /56FAM/CACGTACTGGAGGAGGGTCACCTG      | 3678-3655                    |                      |   |
| Pig actin forward | TAACCGATCCTTTCAAGCATTT               |                              |                      | Krüger et al., 2020 [23]                  |
| Pig actin reverse | TGGTTTCAAAGCTTGCATCATA               |                              |                      |   |
| Pig actin probe   | /5HEX/CGTGGGGATGCTTCCTGAGAAAG        |                              |                      |   |
| Pig GAPDH forward | CCGCGATCTAATGTTCTCTTTC               | 3951-3970                    | NC_010447.5 (396823) | Krüger et al., 2020 [23]                  |
| Pig GAPDH reverse | TTCCTCCGACCTTCACCAT                  | 4022-4001                    |                      |   |
| Pig GAPDH probe   | /5HEX/CAGCCGCGTCCCTGAGACAC           | 3991-3972                    |                      |   |
| PCR1*             |                                      |                              | AM229312             | Takeuchi et al. [25], Kaulitz et al. [26] |
| PERV-C forward    | CTGACCTGGATTAGAACTGG                 | 6606-6625                    |                      |   |
| PERV-C reverse    | ATGTTAGAGGATGGTCCTGG                 | 6867-6886                    |                      | Kaulitz et al. [26]                       |
| PCR6*             |                                      |                              |                      |   |
| PERV-C 2 forward  | CCAGGACCACCAAATAATGG                 | 6435-6454                    |                      |   |
| PERV-C 2 reverse  | AAGTTTTGCCCCCATTTTAGT                | 6924-6944                    |                      |   |
| Real-time PCR*    |                                      |                              |                      |   |
| PERV-C 3 forward  | CCCCAACCCAAGGACCAG                   | 6853-6870                    |                      |   |
| PERV-C 3 reverse  | AAGTTTTGCCCCCATTTTAGT                | 6924-6944                    |                      |   |
| PERV-C probe      | FAM-CTCTAACATAACTTCTGGATCAGACCC-BHQ1 | 6878-6904                    |                      |   |

\*Designation as in Kaulitz et al. [26] denaturation of 10 min at 95°C, followed by 45 amplification cycles (95°C for 15 s, 58°C for 30 s, and 72°C for 40 s) and a final extension at 72°C for 5 min The sensitivity of the real-time PCR was 10 copy (Supplemental Figure 1).



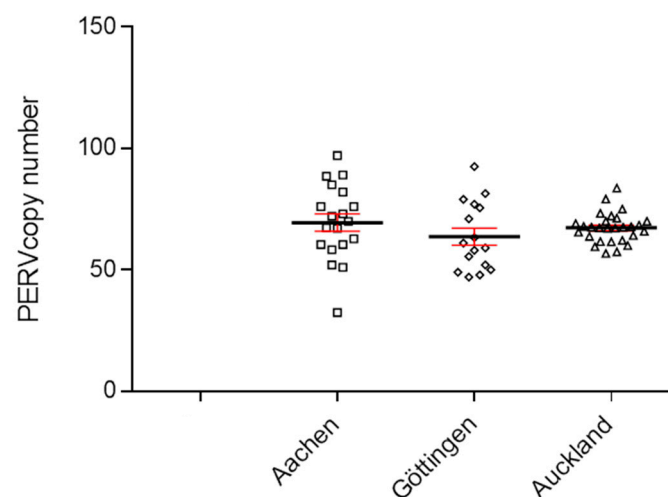
### 2.5. Tests for additional porcine viruses

DNA, RNA and blood from the Auckland Island pigs were used to analyze the presence of different DNA viruses and the RNA virus HEV. DNA was isolated as described above, and RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). Primers and condition used for the PCR or real-time PCR assays have been described for PCMV/PRV [28,29], PCV2 [30], PCV3 [31], PLHV-1, PLHV-2, and PLHV-3 [32] as well as for HEV [32]. The Western blot and ELISA to detect antibodies against HEV using recombinant proteins were described as well [32].

## 3. Results

### 3.1. PERV copy number in the genome of adult Auckland Island pigs

To determine the copy number of PERVs, a ddPCR was developed which used primers binding to a highly conserved region of the polymerase gene (pol) of PERV [23,24]. DNA from 30 Auckland Island pigs, bred in New Zealand, were analyzed using the ddPCR and a PERVpol copy number of around 65 was measured (Figure 1). This copy number was similar to the copy number of Göttingen minipigs and slightly lower compared with Aachen minipigs, investigated in parallel at the same time [24]. Since the copy number of Auckland Island pigs were determined in blood cells, the copy number of Aachen minipigs in spleen tissue, and the copy number of Göttingen minipigs in kidney tissue, it is difficult to compare these figures because the PERV copy number may differ in dependence of the organ (see below and [23,24,33]). Furthermore, the cellular composition of the different tissues was different and all tissues contained blood cells. DNA from kidneys of Göttingen minipigs revealed between 45 and 78 PERVpol copies using GAPDH as a reference and in a second study analyzing 16 Göttingen minipigs a median number of 64 (45-93) was found [24]. When DNA samples from Aachen minipigs were analyzed, a median number of 69 (34-97) was determined [24].



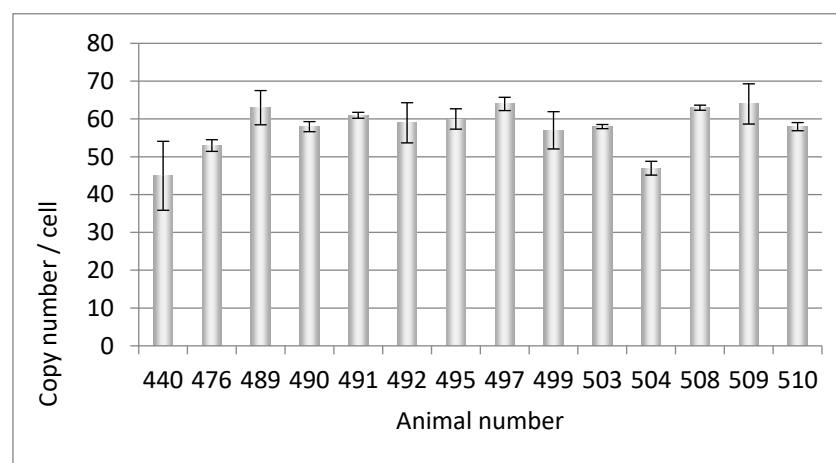
**Figure 1.** Determination of the PERVpol copy number of 30 Auckland Island pigs bred in New Zealand by ddPCR. For comparison the PERVpol copy number of Göttingen minipigs and Aachen minipigs is shown, which were taken from [24]. Porcine GAPDH was chosen as reference gene.

At a later point in time, DNA from another 14 Auckland Island pigs, also bred in New Zealand, were analyzed and copy numbers between 45 and 63 were estimated (Figure 2). This experiment confirmed the previous results shown in Figure 1, but showed a broader range of copy numbers.

### 3.2. Selection of PERV-C negative animals

When the 14 animals tested for the PERV copy number above (Figure 2) and another pig (494) were tested using a conventional PCR, eight out of 15 animals (53%) were PERV-C negative (Table 2). These investigations had the goal to select four PERV-C-free animals from which kidney cells were obtained, which could later be used for the SCNT. PERV-C-negative cells were used for the SCNT (Table 2) and consequentially all cloned Auckland Island piglets were negative for PERV-C in the germline as tested by PCR. It is important to note that the foster mothers were positive for PERV-C and the oocytes were of unknown PERV-C status, most likely PERV-C positive. Therefore, PERV-C was neither transmitted through recipient ooplasm during SCNT procedure nor by the embryo transfer recipient sows.

When Auckland Island pigs of the F0 and F1 generation as well as of later generations were tested by conventional PCR, all animals were negative (not shown). To confirm this, a real-time PCR was established with a limit of detection of 10 copies (Supplementary Figure 1) and all tested animals were PCR negative (no ct, not detected).



**Figure 2.** Determination of the PERVpol copy number of 14 further Auckland Island pigs bred in New Zealand by ddPCR at a later time point. The bars indicate the medium of three samples in one ddPCR reaction.

**Table 2.** Testing of Auckland Island pigs (animals 440-510) and kidney cells from 4 weeks old piglets (w, female, m, male) for different viruses using PCR methods and a Western blot assay and an ELISA (HEV). PERV, median copy number taken from Figure 2.

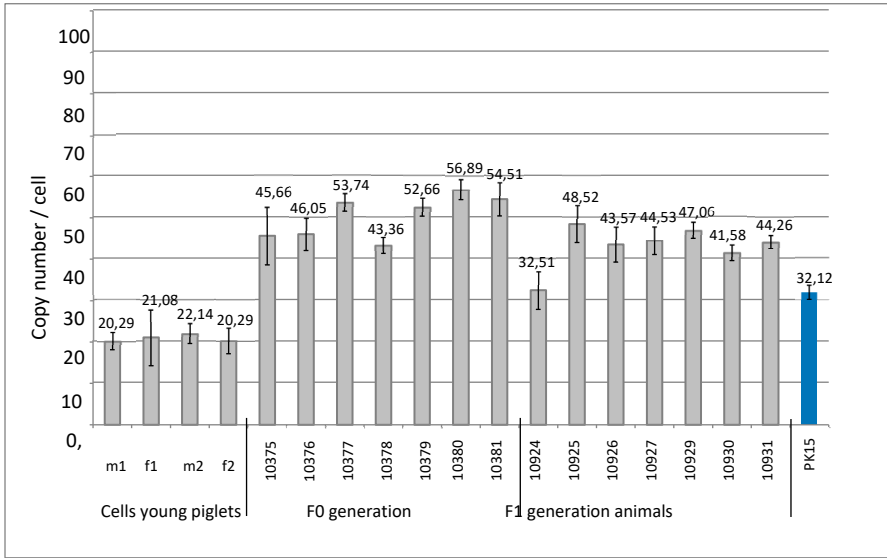
| Pig | Viruses*               |               |                     |            |           |              |               |             |                     |               |
|-----|------------------------|---------------|---------------------|------------|-----------|--------------|---------------|-------------|---------------------|---------------|
|     | PERV<br>copy<br>number | PERV-C<br>PCR | PCMV/P<br>RV<br>PCR | HEV<br>PCR | HEV<br>WB | HEV<br>ELISA | PCV1/2<br>PCR | PCV3<br>PCR | PLHV-<br>1/2<br>PCR | PLHV-3<br>PCR |
|     |                        |               |                     |            |           |              |               |             |                     |               |
| 440 | 45                     | -             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 476 | 53                     | -             | -                   | -          | -         | n.t.         | -             | -           | -                   | +             |
| 489 | 63                     | +             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 490 | 58                     | +             | -                   | -          | -         | n.t.         | -             | -           | -                   | -             |
| 491 | 61                     | +             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 492 | 59                     | +             | -                   | -          | -         | n.t.         | -             | -           | -                   | -             |
| 494 | n.t.                   | +             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 495 | 60                     | -             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 497 | 64                     | -             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 499 | 57                     | -             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 503 | 58                     | -             | -                   | -          | -         | -            | -             | -           | -                   | -             |
| 504 | 47                     | -             | -                   | -          | -         | n.t.         | -             | -           | -                   | -             |

|     |    |   |   |      |      |      |      |   |      |      |
|-----|----|---|---|------|------|------|------|---|------|------|
| 508 | 63 | + | - | -    | -    | n.t. | -    | - | -    | -    |
| 509 | 64 | + | - | -    | -    | n.t. | -    | - | -    | -    |
| 510 | 58 | + | - | -    | -    | n.t. | -    | - | -    | -    |
| f1  | 21 | - | - | n.t. | n.a. | n.a. | n.t. | - | n.t. | n.t. |
| f2  | 20 | - | - | n.t. | n.a. | n.a. | n.t. | - | n.t. | n.t. |
| m1  | 20 | - | - | n.t. | n.a. | n.a. | n.t. | - | n.t. | n.t. |
| m2  | 22 | - | - | n.t. | n.a. | n.a. | n.t. | - | n.t. | n.t. |

\*PERV, porcine endogenous retrovirus; PCMV/PRV, porcine cytomegalovirus/porcine roseolovirus; HEV, hepatitis E virus; PCV, porcine circovirus; PLHV, porcine lymphotropic herpesvirus, n.t., not tested, n.a., not applicable.

3.3. PERV copy number in the genome of cell lines from very young piglets

DNA from four kidney cell lines from young piglets four weeks old which had been selected as PERV-C negative were analyzed for the PERV copy number. A very low copy number of 20-22 copies was found (Figure 3).



**Figure 3.** Determination of the PERVpol copy number of four kidney cell lines from very young Auckland Island pigs, and from the F0 and F1 generation of pigs obtained by somatic cell nuclear transfer (SCNT) using kidney cells from four weeks old piglets. The copy number of PK15 cells was determined as control. m, male, f, female.

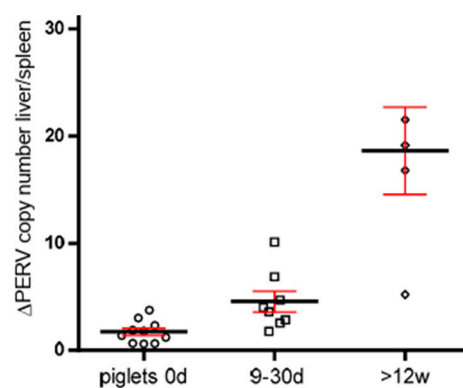
3.4. PERV copy number in the genome of Auckland Island pigs obtained by SCNT

PERV-C-negative kidney cells from four weeks old piglets were used for an SCNT into *in vitro* matured oocytes collected from abattoir derived ovaries from German Landrace/Piértrain hybrid gilts. Ear punches from the animals of the F0 generation at age 416 days generation and kidney cells of the F1 generation at age of 70 days were analyzed for the PERVpol copy number. The copy numbers of the individual pigs ranged from 32 to 57 (Figure 3), but were lower compared with the numbers of the Auckland Island pigs analyzed in the past (Figure 1, Figure 2), and lower than other pig breeds such as the Göttingen minipigs, and the Aachen minipigs [24]. However, since the copy numbers were determined in tissues of different organs, a direct comparison is not possible. PK15 cells were used as internal control in order to be able to compare the results obtained here with previous results measuring PERV copy numbers. Since different copy numbers of integrated PERV were found in different PK15 cell lines [24], here the PK15 cells ordered from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell lines, Braunschweig, Germany (ACC 640), were used and the measured copy number was 32.12 +/- 1.7 (Figure 3), which was in agreement with our previous findings [23,24].



### 3.5. Increase of the PERV copy number with age in non-Auckland Island pigs

In order to obtain additional evidence for the increase of the PERV copy number with age, the copy numbers were determined in two different organs of dead born (day 0), 9-30 days old and older than 12 weeks piglets from non-Auckland Island pigs (German landrace pigs), obtained from the CiMM, Munich. The copy number was determined in the spleen and liver and the difference between the copy number was depicted depending on the age of the animals (Figure 4). This way of presentation best illustrates the result: Differences in the copy number between spleen and liver were well described previously [24], and here it was shown that they increase with age. This indicates that in addition to the copy number in the germ line *de novo* integrations took place in one or the other organ. The *de novo* integrations were not tissue-specific, in some animals the higher copy number was in the spleen, in others in the liver. The difference was larger in older piglets, indicating that the copy number increased in dependence on the age of the animals (Figure 4).



**Figure 4.** Presentation of the differences of the PERV copy number in spleen and liver of non-Auckland Island pigs in dependence on the age of the animals. The PERV copy number was determined in both organs of dead born piglets (day 0), 9-30 days old and 12 weeks old animals using ddPCR and the difference was indicated in dependence on the age.

### 3.6. Further virological characterization of the Auckland Island pigs

In order to characterize the Auckland Island pigs further, PCR-based methods for the detection of PERV-C, as well as the DNA genome of PCMV/PRV, PCV1, PCV2, PCV3, PLHV-1, PLHV-2, and PLHV-3 were performed (Table 2). In addition, a reverse transcriptase (RT) PCR for the RNA genome of HEV were performed (Table 2). The tests for all these viruses were negative, with exception of animal 476. In this animal PLHV-3 was found by PCR. Negative was a Western blot assay and an ELISA screening for antibodies against HEV, confirming that these animals are free of HEV as shown also by PCR. In addition to the Auckland Island pigs, the four kidney cell lines from young piglets four weeks old were tested in addition to PERV-C also for PCMV/PRV, and PCV3 and were found negative (Table 2).

## 4. Discussion

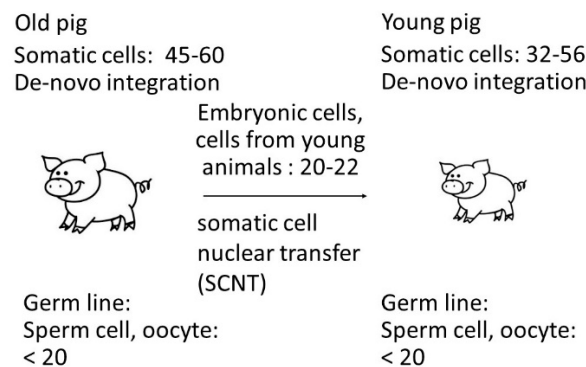
For the first time, a determination of the number of PERV copies in Auckland Island pigs was performed using the ddPCR method. The Auckland Island pigs had been used in the first xenotransplantation trials in New Zealand and Argentina [7,8], they are virologically well characterized [4–6] and are the “cleanest” pigs compared with other pig breeds. Theoretically endogenous retroviruses behave like cellular genes, they are fixed and should be identical in all tissues and cells. However, since PERVs are still active [33] and *de novo* integrations in somatic cells of different organs were observed leading to different copy numbers in different organs (Figure 4), it remains unclear whether all copies are already integrated.

The animals were free of eight porcine viruses, only in one animals PLHV-3 was detected. PLHV-3 does not harm pigs [34]. To note, the closely related PLHV-1, and -2 were not transmitted in preclinical trials transplanting pig hearts into baboons despite the viruses were detected in the donor pigs [9].

In the past it had been shown that the PERV copy number differs in dependence on the pig strain, on the individual animal tested, on the tissue tested, and on the method used (for review see [33]). Auckland Island pigs had been reported previously to contain three to 37 copies using a real-time PCR assay and a light cycler [5] and four to 40 copies were found using a PCR-based limited dilution assay (PLDA) [7]. In another study, relative gene dosages of PERV in Auckland Island pigs ranged from  $2.8 \pm 0.1$  to  $68 \pm 1.6$  at an average of  $17.6 \pm 10.4$  using a real-time PCR [35]. This indicates a generally lower copy number compared to the numbers found here, but some animals had even a higher copy number, up to 68 copies. This was higher than the copy number found in this study. In our study we found that the PERV copy number is much lower in cells from very young piglets used for the SCNT (20-22 copies) compared with the copy number in adult animals (ranging from 32 to 59 copies in the animals obtained from these cells by SCNT) (Figure 3). Unfortunately, in most studies the age of the animals was unknown.

This data confirms that the copy number depends on the method used and probably on the age of the animals. Due to de novo integrations the number of integrated PERV proviruses differs from organ to organ (for review of numerous publications see [24]) and even in different parts of a single organ [23] in living pigs. It is possible that not all detected copies are integrated. The finding that the copy number is lower in cells from very young animals correlates with findings in another study: When analyzing 14-21-day-old neonatal and 1-2-year-old adult Belgian landrace pigs a significant increase in the copy number over time was described [36]. In these animals, a difference between least five copies and 80 copies was reported, indicating that the viruses are active and replicating in the neonatal pig. In Figure 5 a schematic presentation of these events is given. Indicated are the copy numbers found in the somatic cells of the animals and in the cells from young animals used for SCNT measured by ddPCR. Considering that copy numbers in adult animals are the result of *de novo* integrations in somatic cells, it logically follows that the number of proviruses in the germline can be at most 20 (Figure 5).

The fact that the number of integrated proviruses increased by age was also supported by a comparison of the copy number in two different tissues, spleen and liver, of non-Auckland Island pigs (Figure 4). Differences in the number of integrated copies means that in one or the other organ additional copies appeared in addition to the copy number in the germ line. The fact that the difference increases with age indicates that more new copies were integrated with age.



**Figure 5.** Copy number of PERV in somatic cells and in cells used for SCNT indicating increase of the copy number in adult animals.

As shown above, the differences in the copy number of PERV in Auckland Island pigs when measuring by different methods ([5,7,35] and this paper) did not only depend on the method. Based

on our findings the copy number increases due to *de novo* integrations and it depends on the age of the animal which was not always known to us and therefore cannot be taken into account. It would be highly interesting to test the PERV copy number in organs of a single animal over time. Furthermore, the PERV copy number may also depend on the type of tissue and the amount of PBMCs present in this tissue. The probability that *de novo* integrated copies can be found in the PBMCs is high since these cells are proliferating, a prerequisite for integration of gammaretroviruses. PBMCs are prone for release of different PERVs [37] and *de novo* integration evidenced by the presence of integration and release of infectious recombinant PERV-A/C from PBMCs [18,20,21,37]. PERV-A/C have never been found in the germ line of pigs [38]. Future investigations using separated cell populations or *in situ* hybridization experiments may answer this question. Furthermore, treatment of pigs during ontogenesis with antiretroviral drugs may prevent amplification of PERVs.

Auckland Island pigs are descendants of Western pigs brought to the island [1,2]. This was confirmed by phylogenetic analyses of microsatellites and mitochondrial analyses showing that they are more closely related to European pigs than Chinese pigs [3]. The PERV copy number in Western pigs is higher compared with Chinese pigs. By analyzing 63 high-quality porcine whole-genome resequencing data, it was found that the PERV copy numbers in Chinese pigs were lower ( $32.0 \pm 4.0$ ) than in Western pigs ( $49.1 \pm 6.5$ ) [39].

From all methods ever used to count PERV copies in the DNA of pigs, such as Southern blot [40,41], PCR titration [42], real-time PCR [43–48], fluorescence in situ hybridization (FISH) [49], PCR-based limited dilution assay [5], and genome wide sequencing [50], the ddPCR seems to be the most accurate method [33]. For example, using Southern blot analysis about 50 PERV copies were found in certain pig breeds [40,41], but PCR titration [42] or real-time PCR [43–48] detected from one to 98 copies. By using FISH, 19 copies of PERV-A and 13 copies of PERV-B were found in Western pigs [49]. Using genome-wide sequencing, 20 gammaretrovirus copies and 4 betaretrovirus copies were detected in Duroc pigs [50], indicating that sequencing does not detect all integrated viruses.

DdPCR was found to have numerous favorable features in comparison with all other methods including real-time PCR (for review see [33]). One major advantage is that reference standard curves are not required, and bias effects arising from amplification efficiency and PCR inhibitors were reduced. The ddPCR technique provides absolute quantification based on the principles of sample partitioning and Poisson statistics, thus overcoming the normalization and calibrator issues; it has shown increased precision and sensitivity; it is relatively insensitive to PCR inhibitors and directly provides the result of the analysis expressed as number of copies of target per microliter of reaction [51].

Using ddPCR, fifty-nine copies were found when analyzing kidney fibroblasts from multi-transgenic pigs expressing the human transgenes CD46, CD55, CD59, HO1, and A20 [24]. When wild boars in and around Berlin were analyzed using droplet digital PCR, three to 69 PERV copies were found. A lower copy number, but a higher variability of this number was found in wild boars compared to domestic pigs, including minipigs [23].

All PCR-based methods used to estimate the PERV copy number have a common limitation, in that they used primers mostly for the highly conserved pol gene. This allows to detect all PERVs, i.e. PERV-A, PERV-B and PERV-C. However, the detection of this sequence does not mean that there is an intact provirus able to produce infectious virus particles. Therefore, the estimated number of integrated proviruses measured with PCR-based methods tells us nothing concerning the safety of xenotransplantation, e.g., the risk posed by PERVs [52]. Only sequencing the pig genome could give more reliable information. However, sequencing of repetitive, nearly identical sequences is always associated with problems as has been seen with hidden sequences of the human endogenous retrovirus HERV-K [53,54]. In these investigations, in addition to 91 full-length HERV-K proviruses previously found in the human genome draft sequence, hundreds more proviruses were detected. Sequencing the genome of a domestic Duroc pig, 212 endogenous retrovirus sequences were detected, among them 9 PERV-A, 11 PERV-B and 4 betaretrovirus copies [49], a lower number of PERVs compared with other methods [33].

One major result of the work was the elimination of PERV-C. Among the Auckland Island pigs analyzed in the first experiment approximately 53% of the animals were PERV-C-negative (Table 2). PERV-C is an ecotropic virus, infecting only pig cells and therefore does not pose a direct risk for xenotransplantation. However, recombinants between the human-tropic PERV-A and PERV-C have been observed, which are able to infect human cells and which are characterized by higher replication rates [15,17,55]. At present, PERV-A/C were found mainly in minipigs, either released from their PBMCs [18,20,21,38] or present in some organs of minipigs with melanomas [56] or even apparently healthy pigs [57]. Miniature swine may be a unique pig breed as they possess more copies of PERV-C sequence (typically five to nine copies) than many other pig herds (one to three copies [41]), suggesting that PERV-C may be more active in miniature swine [19]. One exception was the finding of PERV-A/C in large farm animals with infectious diseases [22]. However, in all cases PERV-A/C was only found in somatic cells, never in the germ line [58–60].

Due to our selection, there is no risk of PERV-A/C release from Auckland Island pigs generated by SCNT using nuclei from PERV-C-free Auckland Island pigs. These PERV-C free pigs will be a valuable foundation for additional genetic modification in terms of future clinical xenotransplantation.

## 5. Conclusions

Auckland Island pigs had been used repeatedly as donors for pig islet cell transplantation into non-human primates and diabetic patients. In all preclinical and clinical trials no porcine viruses including PERV had been transmitted.

Auckland Island pigs are well characterized, they are free of many potentially pathogenic viruses and have a PERV copy number comparable to other Western pig breeds. The copy number is much lower in cells from very young piglets and in animals obtained by SCNT using nuclei from Auckland Island pig cells. To enhance the virus safety, PERV-C negative animals were selected. In addition, it was shown that eight other xenotransplantation-relevant pig viruses were absent in these animals. Auckland Island pigs have numerous advantages compared with other pig breeds including the size, the health status, the absence of many known viruses and may be, due to the long isolation, also the absence of still unknown potentially zoonotic microorganisms.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Standard curve of the real-time PCR for the detection of PERV-C.

**Author Contributions:** Conceptualization, J.D.; methodology, U.F. and L.K., validation, U.F., L. K. and J.D.; writing—original draft preparation, J.D.; writing—review and editing, U.F. and J.D.; supervision, J.D.; funding acquisition, J.D. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Deutsche Forschungsgemeinschaft, TR127.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data supporting reported results are included in this manuscript.

**Acknowledgments:** We thank Olga Garkavenko and Paul Tan, NZ Xeno Ltd, Auckland, for supplying the DNA and the blood from the New Zealand Auckland Island pigs and for many helpful discussions leading not only to the completion of this manuscript, but also of reference 52. We thank Eckhard Wolf and Barbara Keßler, Chair for Molecular Animal Breeding and Biotechnology and CiMM, Munich, for the DNA of the cells from the very young piglets and the DNA from the Munich Auckland Island pigs and non-Auckland Island pigs as well as for valuable discussions. We thank Bruno Reichart for constant support and advice. We thank Hina Jhelum, Institute of Virology, Free University Berlin, for testing for PERV-C by real-time PCR.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

- Gongora J, Garkavenko, O, Moran C. Origins of Kune Kune and Auckland Island pigs in New Zealand. 7th World Congress on Genetic Applied to Livestock Production, August 19-23, 2002, Montpellier, France
- Robins JH, Matisoo-Smith, E Ross HA. The origins of the feral pigs on the Auckland Islands. *J. Royal Society of New Zealand*, **2003**, 33 (2), 561-569.
- Fan B, Gongora J, Chen Y, Garkavenko O, Li Moran C. Population genetic variability and origin of Auckland Island feral pigs. *J. Royal Society of New Zealand*, **2005**, 35(3), 279-285.
- Garkavenko O, Muzina M, Muzina Z, Powels K, Elliott RB, Croxson MC. Monitoring for potentially xenozoonotic viruses in New Zealand pigs. *J Med Virol*. **2004**, 72(2), 338-344.
- Garkavenko O, Wynyard S, Nathu D, Simond D, Muzina M, Muzina Z, Scobie L, Hector RD, Croxson MC, Tan P, Elliott BR. Porcine endogenous retrovirus (PERV) and its transmission characteristics: a study of the New Zealand designated pathogen-free herd. *Cell Transplant*. **2008**, 17(12), 1381-1388.
- Garkavenko O, Dieckhoff B, Wynyard S, Denner J, Elliott RB, Tan PL, Croxson MC. Absence of transmission of potentially xenotic viruses in a prospective pig to primate islet xenotransplantation study. *J Med Virol*. **2008**, 80(11), 2046-2052.
- Wynyard S, Nathu D, Garkavenko O, Denner J, Elliott R. Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. *Xenotransplantation*. **2014**, 21(4), 309-323.
- Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. *Virus Res*. **2017**, 227, 34-40.
- Denner J, Längin M, Reichart B, Krüger L, Fiebig U, Mokelke M, Radan J, Mayr T, Milusev A, Luther F, Sorvillo N, Rieben R, Brenner P, Walz C, Wolf E, Roshani B, Stahl-Hennig C, Abicht JM. Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival. *Sci Rep*. **2020**, 10(1):17531
- Yamada K, Tasaki M, Sekijima M, Wilkinson RA, Villani V, Moran SG, Cormack TA, Hanekamp IM, Hawley RJ, Arn JS, Fishman JA, Shimizu A, Sachs DH. Porcine cytomegalovirus infection is associated with early rejection of kidney grafts in a pig to baboon xenotransplantation model. *Transplantation*. **2014**, 98(4):411-418.
- Sekijima M, Waki S, Sahara H, Tasaki M, Wilkinson RA, Villani V, Shimatsu Y, Nakano K, Matsunari H, Nagashima H, Fishman JA, Shimizu A, Yamada K. Results of life-supporting galactosyltransferase knockout kidneys in cynomolgus monkeys using two different sources of galactosyltransferase knockout Swine. *Transplantation*. **2014**, 98(4):419-426.
- Denner J. Reduction of the survival time of pig xenotransplants by porcine cytomegalovirus. *Virol J*. **2018**, 15(1):171.
- Griffith BP, Goerlich CE, Singh AK, Rothblatt M, Lau CL, Shah A, Lorber M, Grazioli A, Saharia KK, Hong SN, Joseph SM, Ayares D, Mohiuddin MM. Genetically Modified Porcine-to-Human Cardiac Xenotransplantation. *N Engl J Med*. **2022**, 387(1):35-44.
- Mohiuddin MM, Singh AK, Scobie L, Goerlich CE, Grazioli A, Saharia K, Crossan C, Burke A, Drachenberg C, Oguz C, Zhang T, Lewis B, Hershsfeld A, Sentz F, Tatarov I, Mudd S, Braileanu G, Rice K, Paolini JF, Bondensgaard K, Vaught T, Kuravi K, Sorrells L, Dandro A, Ayares D, Lau C, Griffith BP. Graft dysfunction in compassionate use of genetically engineered pig-to-human cardiac xenotransplantation: a case report. *Lancet*. 2023, S0140-6736(23)00775-4.
- Denner J, Specke V, Thiesen U, Karlas A, Kurth R. Genetic alterations of the long terminal repeat of an ecotropic porcine endogenous retrovirus during passage in human cells. *Virology*. 2003, 314(1):125-133.
- Karlas A, Irgang M, Votteler J, Specke V, Ozel M, Kurth R, Denner J. Characterisation of a human cell-adapted porcine endogenous retrovirus PERV-A/C. *Ann Transplant*. 2010, 15(2):45-54.
- Harrison I, Takeuchi Y, Bartosch B, Stoye JP. Determinants of high titer in recombinant porcine endogenous retroviruses. *J Virol*. 2004, 78(24):13871-1389
- Wilson CA, Wong S, Muller J, Davidson CE, Rose TM, Burd P. Type C retrovirus released from porcine primary peripheral blood mononuclear cells infects human cells. *J Virol*. 1998, 72(4):3082-3087.
- Denner J, Schuurmann KJ. High prevalence of recombinant porcine endogenous retroviruses (PERV-A/Cs) in minipigs: a review on origin and presence. *Viruses*. 2021, 13:1869.
- Krüger L, Kristiansen Y, Reuber E, Möller L, Laue M, Reimer C, Denner J. A comprehensive strategy for screening for xenotransplantation-relevant viruses in a second isolated population of Göttingen Minipigs. *Viruses*. 2019, 12(1):38



21. Halecker S, Krabben L, Kristiansen Y, Krüger L, Möller L, Becher D, Laue M, Kaufer B, Reimer C, Denner J. Rare isolation of human-tropic recombinant porcine endogenous retroviruses PERV-A/C from Göttingen minipigs. *Virol J.* 2022, 19(1):30
22. Pal N, Baker R, Schalk S, Scobie L, Tucker AW, Opriessnig T. Detection of porcine endogenous retrovirus (PERV) viremia in diseased versus healthy US pigs by qualitative and quantitative real-time RT-PCR. *Transbound Emerg. Dis.* 2011, 58, 344–351.
23. Krüger L, Stillfried M, Prinz C, Schröder V, Neubert LK, Denner J. Copy Number and Prevalence of Porcine Endogenous Retroviruses (PERVs) in German Wild Boars. *Viruses.* 2020, 12(4):419.
24. Fiebig U, Fischer K, Bähr A, Runge C, Schnieke A, Wolf E, Denner J. Porcine endogenous retroviruses: Quantification of the copy number in cell lines, pig breeds, and organs. *Xenotransplantation.* 2018, 25(4):e12445.
25. Takeuchi Y, Patience C, Magre S, Weiss RA, Banerjee PT, Le Tissier P, Stoye JP (1998) Host range and interference studies of three classes of pig endogenous retrovirus. *J Virol* 1998, 72:9986-9991
26. Kaulitz D, Mihica D, Adlhoch C, Semaan M, Denner J. Improved pig donor screening including newly identified variants of porcine endogenous retrovirus-C (PERV-C). *Arch Virol.* 2013, 158(2):341-348.
27. Yang, L.; Güell, M.; Niu, D.; George, H.; Leshia, E.; Grishin, D.; Aach, J.; Shrock, E.; Xu, W.; Poci, J.; et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* 2015, 350, 1101–1104.
28. Morozov VA, Plotzki E, Rotem A, Barkai U, Denner J. Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses. *Xenotransplantation.* 2016, 23(6), 490-496.
29. Morozov VA, Morozov AV, Denner J. New PCR diagnostic systems for the detection and quantification of porcine cytomegalovirus (PCMV). *Arch Virol.* 2016, 161(5), 1159-1168.
30. Heinze, J.; Plotzki, E.; Denner, J. Virus Safety of Xenotransplantation: Prevalence of Porcine Cytocivirus 2 (PCV2) in Pigs. *Ann. Virol. Res.* 2016, 2, 1023.
31. Prinz, C.; Stillfried, M.; Neubert, L.K.; Denner, J. Detection of PCV3 in German wild boars. *Virol. J.* 2019, 16, 25.
32. Morozov, V.A.; Morozov, A.V.; Rotem, A.; Barkai, U.; Bornstein, S.; Denner, J. Extended Microbiological Characterization of Göttingen Minipigs in the Context of Xenotransplantation: Detection and Vertical Transmission of Hepatitis E Virus. *PLoS ONE* 2015, 10, e0139893.
33. Denner J. How Active Are Porcine Endogenous Retroviruses (PERVs)? *Viruses.* 2016, 8(8):215.
34. Denner J. Porcine Lymphotropic Herpesviruses (PLHVs) and Xenotransplantation. *Viruses* 2021, 13(6), 1072.
35. Wynyard S, Garkavenko O, Elliot R. Multiplex high resolution melting assay for estimation of Porcine Endogenous Retrovirus (PERV) relative gene dosage in pigs and detection of PERV infection in xenograft recipients. *J Virol Methods.* 2011, 175(1):95-100.
36. Mourad NI, Crossan C, Cruikshank V, Scobie L, Gianello P. Characterization of porcine endogenous retrovirus expression in neonatal and adult pig pancreatic islets. *Xenotransplantation.* 2017, 24(4).
37. Tacke SJ, Specke V, Denner J. Differences in release and determination of subtype of porcine endogenous retroviruses produced by stimulated normal pig blood cells. *Intervirology.* 2003, 46(1), 17-24.
38. Bartosch B, et al. Evidence and consequence of porcine endogenous retrovirus recombination. *J. Virol.* 2004; 78, 13880–13890.
39. Chen JQ, Zhang MP, Tong XK, Li JQ, Zhang Z, Huang F, Du HP, Zhou M, Ai HS, Huang LS. Scan of the endogenous retrovirus sequences across the swine genome and survey of their copy number variation and sequence diversity among various Chinese and Western pig breeds. *Zool Res.* 2022, 43(3), 423-441.
40. Le Tissier, P.; Stoye, J.P.; Takeuchi, Y.; Patience, C.; Weiss, R.A. Two sets of human-tropic pig retrovirus. *Nature* 1997, 389, 681–682.
41. Patience, C.; Takeuchi, Y.; Weiss, R.A. Infection of human cells by an endogenous retrovirus of pigs. *Nat. Med.* 1997, 3, 282–286.
42. Patience, C.; Switzer, W.M.; Takeuchi, Y.; Griffiths, D.J.; Goward, M.E.; Heneine, W.; Stoye, J.P.; Weiss, R.A. Multiple groups of novel retroviral genomes in pigs and related species. *J. Virol.* 2001, 75, 2771–2775.
43. Liu, G.; Li, Z.; Pan, M.; Ge, M.; Wang, Y.; Gao, Y. Genetic prevalence of porcine endogenous retrovirus in Chinese experimental miniature pigs. *Transplant. Proc.* 2011, 43, 2762–2769.
44. Lee, D.; Lee, J.; Yoon, J.K.; Kim, N.Y.; Kim, G.W.; Park, C.; Oh, Y.K.; Kim, Y.B. Rapid determination of PERV copy number from porcine genomic DNA by real-time polymerase chain reaction. *Anim. Biotechnol.* 2011, 22, 175–180.



45. Yoon, J.K.; Choi, J.; Lee, H.J.; Cho, Y.; Gwon, Y.D.; Jang, Y.; Kim, S.; Choi, H.; Lee, J.H.; Kim, Y.B. Distribution of Porcine Endogenous Retrovirus in Different Organs of the Hybrid of a Landrace and a Jeju Domestic Pig in Korea. *Transplant. Proc.* 2015, 47, 2067–2071.
46. Zhang, P.; Yu, P.; Wang, W.; Zhang, L.; Li, S.; Bu, H. An effective method for the quantitative detection of porcine endogenous retrovirus in pig tissues. *In Vitro Cell Dev. Biol. Anim.* 2010, 46, 408–410.
47. Quereda, J.J.; Herrero-Medrano, J.M.; Abellaneda, J.M.; García-Nicolás, O.; Martínez-Alarcón, L.; Pallarés, F.J.; Ramírez, P.; Muñoz, A.; Ramis, G. Porcine endogenous retrovirus copy number in different pig breeds is not related to genetic diversity. *Zoonoses Public Health* 2012, 59, 401–407.
48. Mang, R.; Maas, J.; Chen, X.; Goudsmit, J.; van der Kuyl, A.C. Identification of a novel type C porcine endogenous retrovirus: Evidence that copy number of endogenous retroviruses increases during host inbreeding. *J. Gen. Virol.* 2001, 82, 1829–1834.
49. Lee, J.H.; Webb, G.C.; Allen, R.D.; Moran, C. Characterizing and mapping porcine endogenous retroviruses in Westran pigs. *J. Virol.* 2002, 76, 5548–5556.
50. Groenen MA, Archibald AL, Uenishi H, Tuggle CK, Takeuchi Y, Rothschild MF, Rogel-Gaillard C, Park C, Milan D, Megens HJ, Li S, Larkin DM, Kim H, Frantz LA, Caccamo M, Ahn H, Aken BL, Anselmo A, Anthon C, Auvin L, Badaoui B, Beattie CW, Bendixen C, Berman D, Blecha F, Blomberg J, Bolund L, Bosse M, Botti S, Bujie Z, Bystrom M, Capitanu B, Carvalho-Silva D, Chardon P, Chen C, Cheng R, Choi SH, Chow W, Clark RC, Clee C, Crooijmans RP, Dawson HD, Dehais P, De Sapio F, Dibbits B, Drou N, Du ZQ, Eversole K, Fadista J, Fairley S, Faraut T, Faulkner GJ, Fowler KE, Fredholm M, Fritz E, Gilbert JG, Giuffra E, Gorodkin J, Griffin DK, Harrow JL, Hayward A, Howe K, Hu ZL, Humphray SJ, Hunt T, Hornshøj H, Jeon JT, Jern P, Jones M, Jurka J, Kanamori H, Kapetanovic R, Kim J, Kim JH, Kim KW, Kim TH, Larson G, Lee K, Lee KT, Leggett R, Lewin HA, Li Y, Liu W, Loveland JE, Lu Y, Lunney JK, Ma J, Madsen O, Mann K, Matthews L, McLaren S, Morozumi T, Murtaugh MP, Narayan J, Nguyen DT, Ni P, Oh SJ, Onteru S, Panitz F, Park EW, Park HS, Pascal G, Paudel Y, Perez-Enciso M, Ramirez-Gonzalez R, Reecy JM, Rodriguez-Zas S, Rohrer GA, Rund L, Sang Y, Schachtschneider K, Schraiber JG, Schwartz J, Scobie L, Scott C, Searle S, Servin B, Southey BR, Sperber G, Stadler P, Sweedler JV, Tafer H, Thomsen B, Wali R, Wang J, Wang J, White S, Xu X, Yerle M, Zhang G, Zhang J, Zhang J, Zhao S, Rogers J, Churcher C, Schook LB. Analyses of pig genomes provide insight into porcine demography and evolution. *Nature* 2012, 491, 393–398.
51. Pinheiro, L.B.; Coleman, V.A.; Hindson, C.M.; Herrmann, J.; Hindson, B.J.; Bhat, S.; Emslie, K.R. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal. Chem.* 2012, 84, 1003–1011.
52. Denner J. What does the PERV copy number tell us? *Xenotransplantation*. 2022, 2:e12732.
53. Subramanian RP, Wildschutte JH, Russo C, Coffin JM. Identification, characterization, and comparative genomic distribution of the HERV-K (HML-2) group of human endogenous retroviruses. *Retrovirology*. 2011, 8:90.
54. Contreras-Galindo R, Kaplan MH, He S, Contreras-Galindo AC, Gonzalez-Hernandez MJ, Kappes F, Dube D, Chan SM, Robinson D, Meng F, Dai M, Gitlin SD, Chinnaiyan AM, Omenn GS, Markovitz DM. HIV infection reveals widespread expansion of novel centromeric human endogenous retroviruses. *Genome Res.* 2013, 23(9), 1505–1513.
55. Denner J. Recombinant porcine endogenous retroviruses (PERV-A/C): a new risk for xenotransplantation? *Adv. Virol.* 2008, 153, 1421–1426.
56. Dieckhoff B, Puhlmann J, Büscher K, Hafner-Marx A, Herbach N, Bannert N, Büttner M, Wanke R, Kurth R, Denner J. Expression of porcine endogenous retroviruses (PERVs) in melanomas of Munich miniature swine (MMS) Trol. *Vet. Microbiol.* 2007, 123, 53–68.
57. Bittmann I, Mihica D, Plesker R, Denner J. Expression of porcine endogenous retroviruses (PERV) in different organs of a pig. *Virology*. 2012, 433, 329–336.
58. Scobie L, Taylor S, Wood JC, Suling KM, Quinn G, Meikle S, Patience C, Schuurman HJ, Onions DE. Absence of replication-competent human-tropic porcine endogenous retroviruses in the germ line DNA of inbred miniature Swine. *J. Virol.* 2004, 78, 2502–2509.
59. Wood JC, Quinn G, Suling KM, Oldmixon BA, Van Tine BA, Cina R, Arn S, Huang CA, Scobie L, Onions DE, Sachs DH, Schuurman HJ, Fishman JA, Patience C. Identification of exogenous forms of human-tropic porcine endogenous retrovirus in miniature Swine. *J. Virol.* 2004, 78, 2494–2501.

60. Martin SI, Wilkinson R, Fishman JA. Genomic presence of recombinant porcine endogenous retrovirus in transmitting miniature swine. *Virology*. 2006; 3, 91.

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.